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Nef-Induced Major Histocompatibility Complex Class I Down-Regulation Is Functionally Dissociated from Its Virion Incorporation, Enhancement of Viral Infectivity, and CD4 Down-Regulation

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The N-terminal alpha-helix domain of the human immunodeficiency virus type 1 (HIV-1) Nef protein plays important roles in enhancement of viral infectivity, virion incorporation of Nef, and the down-regulation of major histocompatibility complex class I (MHC-I) expression on cell surfaces. In this study, we demonstrated that Met 20 in the alpha-helix domain was indispensable for the ability of Nef to modulate MHC-I expression but not for other events. We also showed that Met 20 was unnecessary for the down-regulation of CD4. These findings indicate that the region governing MHC-I down-regulation is proximate in the alpha-helix domain but is dissociated functionally from that determining enhancement of viral infectivity, virion incorporation of Nef, and CD4 down-regulation.

The *nef* gene is unique to primate lentiviruses and is shown to be associated with their pathogenesis. In macaque monkeys infected with simian immunodeficiency virus, its *nef* gene is required for maintaining high viral loads and inducing $CD4^+$-lymphocyte depletion (16, 27). Similar findings have been obtained from a severe combined immunodeficiency-hu mouse model infected with human immunodeficiency virus type 1 (HIV-1) (12, 15) and HIV-1-transgenic mice (13). Recent findings that the *nef* gene of HIV-1 derived from long-term nonprogressive carriers is truncated suggest the requirement of the intact *nef* gene for disease progression (18, 21, 25).

Functional characterization of Nef protein in vitro has shown that Nef (i) down-regulates the expression of CD4 and major histocompatibility complex class I (MHC-I) molecules, (ii) affects cellular signal transduction pathways, and (iii) enhances viral infectivity (for review, see references 14 and 28). It has also been reported that Nef is incorporated into the virions, where it is cleaved by the viral protease between amino acids 57 and 58 (24, 30), although the biological implication of virion incorporation of Nef remains unknown (9, 22, 23). These effects of Nef are associated with its membrane anchoring, and N-terminal myristoylation of Nef is a determinant for the anchoring (14, 28). However, studies on other myristoylated proteins have indicated that myristoylation alone is not sufficient to stably anchor a protein into the membrane (26).

The N-terminal residues 6 to 22 adopt an alpha-helix structure (5). An N-terminal Arg-rich motif (I^{16}RERMRR^{22}), which is well conserved among the Nef proteins of major HIV-1 strains, is required for association with a protein complex containing Lck and serine kinases and for optimal viral infectivity in resting peripheral blood mononuclear cells (PBMCs) but is dispensable for down-regulation of CD4 (6). It has been shown that bipartite motifs in the N terminus of Nef (K^4xxK^7 and R^{17}ERMRR^{22}) play important roles in virion incorporation of Nef and in viral infectivity (29). Recently, Mangasarian et al. have shown that a mutant Nef protein in which residues 17 to 26 have been deleted fails to down-regulate MHC-I while retaining the ability to modulate CD4 levels (20), in agreement with previous reports (3, 6, 8, 10).

In this study, we investigated the functional role of the conserved methionine at position 20, which is present in the amphipathic alpha-helix domain of Nef protein. For this purpose, the second ATG codon, coding for Met 20, in the *nef* gene of HIV-1_{NL-432} (wild type [WT]) provirus clone pNL-432 (1) was mutated into GCG coding for Ala by using an LA PCR in vitro mutagenesis kit (Takara, Kusatsu, Japan). The mutant clone was designated pNL-M20A. This single amino acid exchange maintains hydrophobicity at this position. Considering the possibility that hydrophobicity could be important for proper function of the motif, the ATG codon was also changed into AGG coding for Arg to generate a pNL-M20R mutant. As controls, a Nef-defective mutant, pNL-Xh (referred to herein as Xh), having a frame shift at a *Xho*I site (2) and another Nef mutant, pNL-M1T (referred to herein as M1T), which lacks expression of Nef because of an alteration of the first ATG codon to ACC, were used. The mutations are summarized in Fig. 1A.

We initially examined the expression of the Nef protein in cells. HeLa cells were transfected with each proviral DNA clone by the calcium phosphate coprecipitation method (31). The lysates of transfected HeLa cells were quantified for the amount of p24 capsid antigen by a p24 antigen enzyme-linked immunosorbent assay kit (Cellular Products, Buffalo, N.Y.). The lysates containing an equal amount of the viral antigen were subjected to electrophoresis in a sodium dodecyl sulfate-gradient polyacrylamide gel. The separated proteins were blotted onto nitrocellulose membranes, treated with antibodies to HIV-1 proteins, and visualized using an ECL system (Amer-

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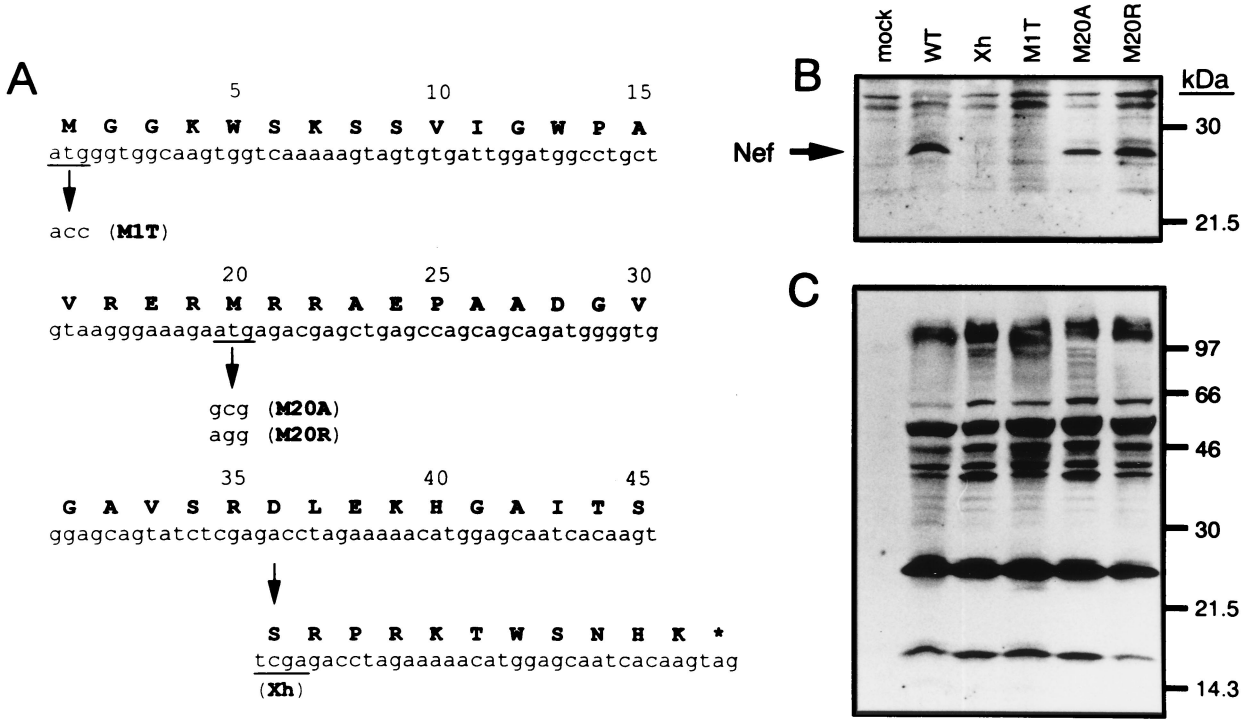

FIG. 1. Analysis of cellular expression of Nef proteins. (A) Sequences of HIV-1 *nef* mutants used here are shown. The N-terminal 45 residues of Nef and the corresponding nucleic acid sequence are depicted. The asterisk indicates the stop codon. (B and C) Lysates containing an equal amount of p24 capsid antigen were prepared from HeLa cells transfected with the respective proviral DNA clones and were analyzed by Western blotting with a rabbit anti-Nef antiserum (B) and a human anti-HIV-1 antiserum (C).

sham, Little Chalfont, Buckinghamshire, United Kingdom). Anti-Nef antisera were provided by R. Swanstrom (through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases [NIAID], National Institutes of Health [NIH]) and Y. Takebe (National Institute of Infectious Diseases, Tokyo, Japan). Total virus antigens were detected by an anti-HIV-1 antiserum. The results of this experiment showed that mutations of Met 20 to Ala and Arg did not much affect the expression of the 27-kDa Nef protein, which was detected in pNL-432-transfected cells (Fig. 1B). The Xh- or M1T-transfected HeLa cells did not express the Nef protein (Fig. 1B). In addition, similar levels of the major viral antigens were observed among the cells transfected with the WT or the Nef mutants (Fig. 1C).

To determine whether Met 20 of the conserved amphipathic motif plays a role in virion incorporation of Nef, we examined the ability of the WT and mutant Nef proteins to be incorporated into virions. For this purpose, the lysates of the WT and Nef mutant viruses produced from HeLa cells transfected with the respective proviral DNA plasmids were subjected to Western blotting analysis as described above. Both a full-length Nef and its C-terminal core, which is a 20-kDa product cleaved in the virion by the viral protease, were demonstrated in the pNL-M20A and pNL-M20R viruses as well as the WT virus (Fig. 2A). These Nef proteins were not detected in Xh and M1T viruses (Fig. 2A). No major difference in the viral proteins was observed among the WT and its Nef variants (Fig. 2B). These results indicated the dispensability of Met 20 for virion incorporation of Nef.

We then investigated the effect of mutations in the *nef* gene on viral infectivity in PBMCs. Resting PBMCs were infected with appropriate amounts of viruses which were adjusted by reverse transcriptase (RT) activity (32). Two days after infection, infected PBMCs were stimulated for 2 days by the addition of 0.5 μg of phytohemagglutinin P (Difco, Detroit, Mich.) per ml. The cells were maintained by exchanging every 2 days half the volume with culture medium consisting of RPMI 1640 with 10% fetal bovine serum, L-glutamine, antibiotics, and 50 U of recombinant human interleukin-2 (Serotec, Oxford, United Kingdom) per ml. The kinetics of viral replication were monitored by measuring RT activity in the culture supernatants. When a relatively large amount of virus (10^5 cpm of RT activity) was used, Nef-defective viruses Xh and M1T showed delayed kinetics and about a three-times-lower peak of replication than the WT, pNL-M20A, and pNL-M20R viruses (Fig. 3A). A decrease of the amount of input viruses to one-fifth (2×10^4 cpm of RT activity) reduced the growth of the Xh and M1T viruses to undetectable levels (Fig. 3B), although the WT, pNL-M20A, and pNL-M20R viruses replicated comparably (Fig. 3B). The slightly delayed growth kinetics of pNL-M20A (Fig. 3B) could be due to the reduced expression of Nef of this mutant (Fig. 1B). These results suggested that mutations in Met 20 of Nef did not much affect viral infectivity. To further address the potential contribution of Met 20 to viral infectivity, the WT and Nef mutant viruses were examined for single-round infectivity by MAGI assay (17). MAGI cells were plated in 96-well plates and infected in triplicate with serially diluted viruses in a total of 200 μl of culture medium containing 20 μg of DEAE-dextran (Sigma, St. Louis, Mo.) per ml. Infected cells were incubated for 2 days at 37°C, fixed, and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Blue cells were counted as infected cells. It was demonstrated that the infectivity of the pNL-M20A and pNL-M20R viruses was comparable to that of WT virus (Fig. 3C). The Xh and M1T

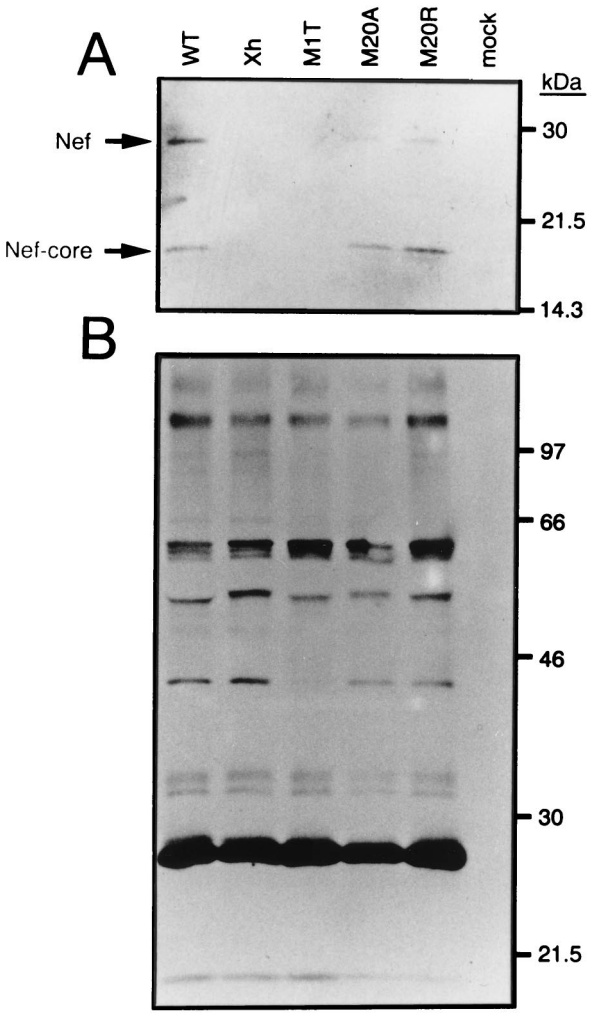

FIG. 2. Incorporation of Nef proteins into virions. HIV-1 WT and *nef* mutant viruses were prepared in HeLa cells transfected with the respective proviral DNA clones. Virus lysates containing an equal amount of p24 capsid antigen were analyzed by Western blotting with a rabbit anti-Nef antiserum (A) and a human anti-HIV-1 antiserum (B).

viruses showed about 10-times-lower infectivity than WT virus (Fig. 3C). Taken together, the results show that Met 20 is dispensable for cellular expression of Nef and its incorporation into virions and for the enhancing the effects of Nef on both single and multiple cycles of HIV-1 infection.

In the course of our study, Mangasarian et al. reported that a mutant Nef protein with a deletion of the N-terminal alpha-helix domain at residues 17 to 26 fails to down-regulate MHC-I while retaining the ability to modulate the CD4 level (20). We therefore examined whether Met 20 contributes to the ability of Nef to down-regulate MHC-I expression on the cell surface. Since CEM-GFP cells contain an HIV-1 long terminal repeat-driven green fluorescence protein (GFP) cDNA and GFP expression is inducible by Tat (11), we used it for measuring directly the level of MHC-I expression on HIV-1-infected cells. The CEM-GFP cell line was provided from J. Corbeil through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. CEM-GFP cells (10^5) were infected with 5×10^5 cpm of RT from the WT or the Nef mutant viruses prepared from transfected HeLa cells. When syncytium formation was observed a few days after infection, the cells were treated with an R-phycoerythrin (RPE)-conjugated mouse anti-MHC-I monoclonal antibody (W6/32; Dako, Glostrup, Denmark) at 4°C for 1 h. The cells were washed and fixed with 1% formaldehyde, and the fluorescence intensity for GFP and MHC-I was detected by a FACSCalibur (Becton Dickinson, Mountain View, Calif.). As shown in Fig. 4A, in the case of WT virus infection, down-regulation of MHC-I expression on GFP-positive cells was obvious, and especially the fluorescence intensity for GFP was reversely correlated with the level of MHC-I expression (Fig. 4A). In sharp contrast, infection with either pNL-M20A or pNL-M20R scarcely down-regulated MHC-I expression on GFP-positive cells; the same was true for the Xh and M1T Nef-defective mutants (Fig. 4A). The level of MHC-I expressed on each GFP-positive cell population was calculated as geometric mean fluorescence using CELLQuest software (Becton Dickinson), and the relative

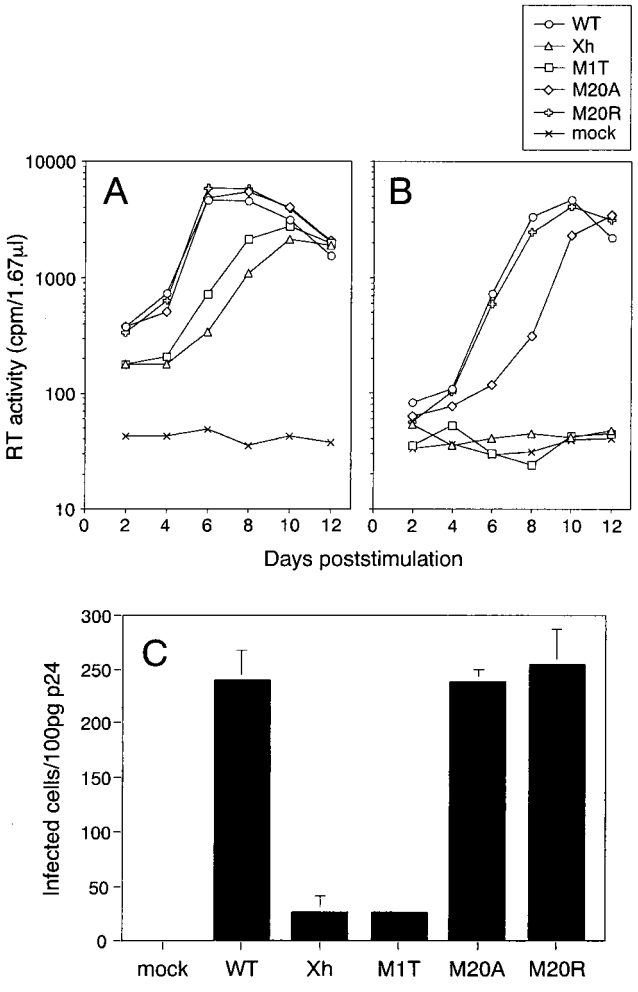

FIG. 3. Analysis of the infectivity of the WT and *nef* mutants of HIV-1. (A and B) Replication kinetics of the viruses in PBMCs. Viruses were obtained from HeLa cells transfected with WT and various *nef* mutant proviral clones. Unstimulated PBMCs (10^5) were infected with 1×10^5 (A) and 2×10^4 (B) cpm of RT from viruses, and 2 days later, cells were stimulated by the addition of 0.5 μg of phytohemagglutinin P per ml. A half volume of the culture supernatants was harvested and refed with the same volume of culture medium with 50 U of recombinant human interleukin-2 per ml every 2 days. Kinetics of RT production in the culture supernatants are indicated. (C) Single-round infectivity of the viruses. Infectivity was determined by counting blue foci of X-Gal-treated MAGI cells 2 days after inoculation with the viruses. Averages and standard deviations of triplicate titrations of the same viral stock are shown.

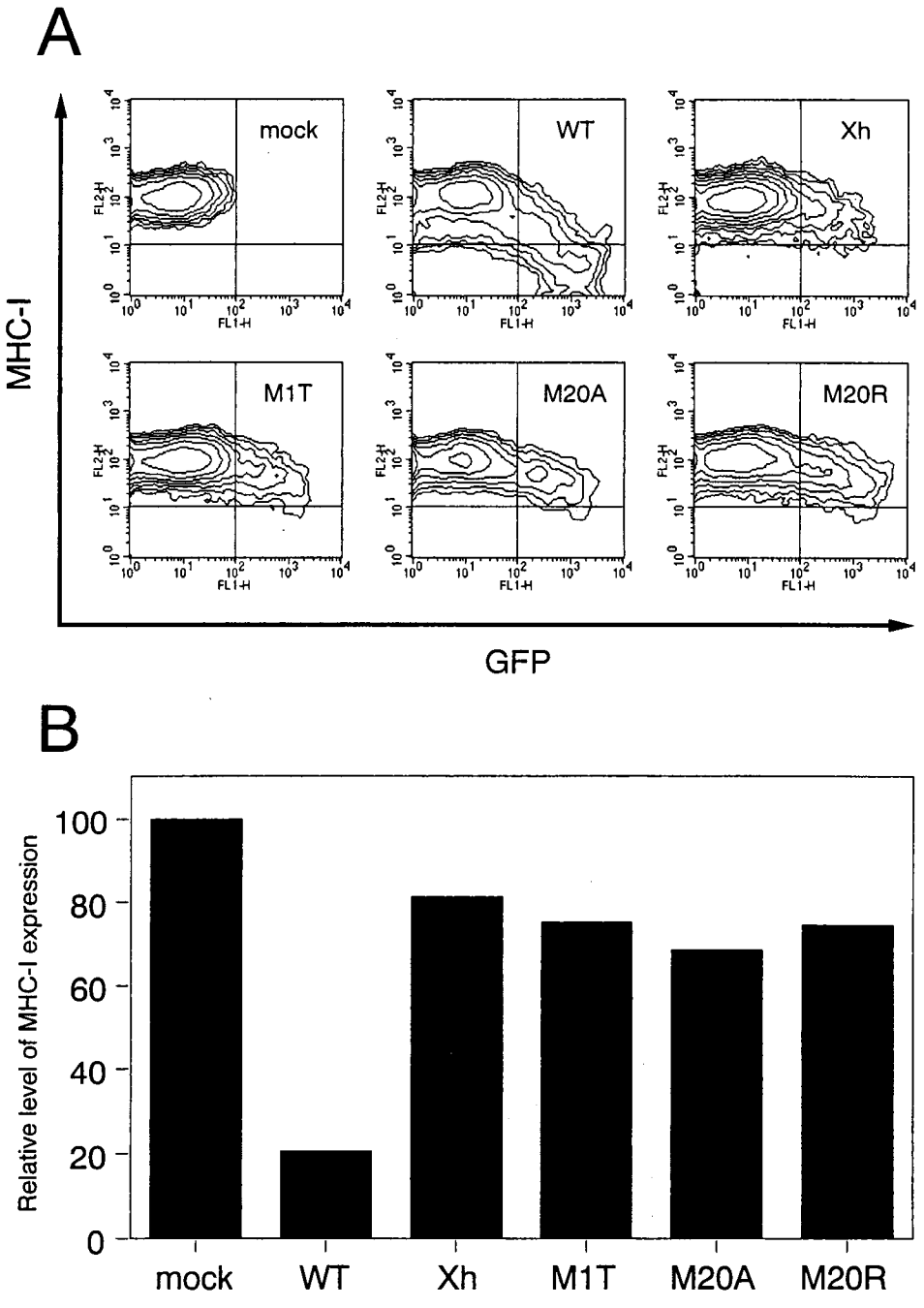

FIG. 4. Effect of mutations in Nef protein on MHC-I down-regulation on the surfaces of HIV-1-infected cells. (A) CEM-GFP cells (10^5) were infected with 5×10^5 cpm of RT from each virus obtained from transfected HeLa cells, and 3 to 5 days later the cells were reacted with an RPE-labeled anti-MHC-I antibody at 4°C for 1 h. The cells were washed, fixed with 1% formaldehyde, and analyzed for fluorescence intensity by flow cytometry. (B) The level of MHC-I expression on the GFP-positive population in virus-inoculated CEM-GFP cells was evaluated as geometric mean fluorescence using CELLQuest software (Becton Dickinson).

level of MHC-I expression compared with that on mock-infected cells is shown (Fig. 4B). The level of MHC-I expression on WT-infected cells was 20% of that on uninfected cells, while that on any of the mutant-infected cells was about 70 to 80% of that on uninfected cells. From this result, it is clearly demonstrated that Met 20 in the N-terminal alpha-helix domain is essential for the ability of Nef to modulate MHC-I expression. We further determined whether Met 20 is important for the ability of Nef to down-regulate CD4 expression. For an assay of CD4 down-regulation, we made other DNA constructs lacking expression of Env and Vpu, which affect the CD4 level. The *Bam*HI-*Nco*I DNA fragments of the mutants containing the mutated *nef* genes (M1T, M20A, and M20R) were inserted into the corresponding region of pNL43-Ude1.K1, which is defective for both *vpu* and *env* (7). These mutants were designated pNL43-Ude1.K1.nM1T, pNL43-Ude1.K1.nM20A, and pNL43-Ude1.K1.nM20R, respectively. The envelope glycoproteins of vesicular stomatitis virus (VSV-G)–HIV-1 pseudo-

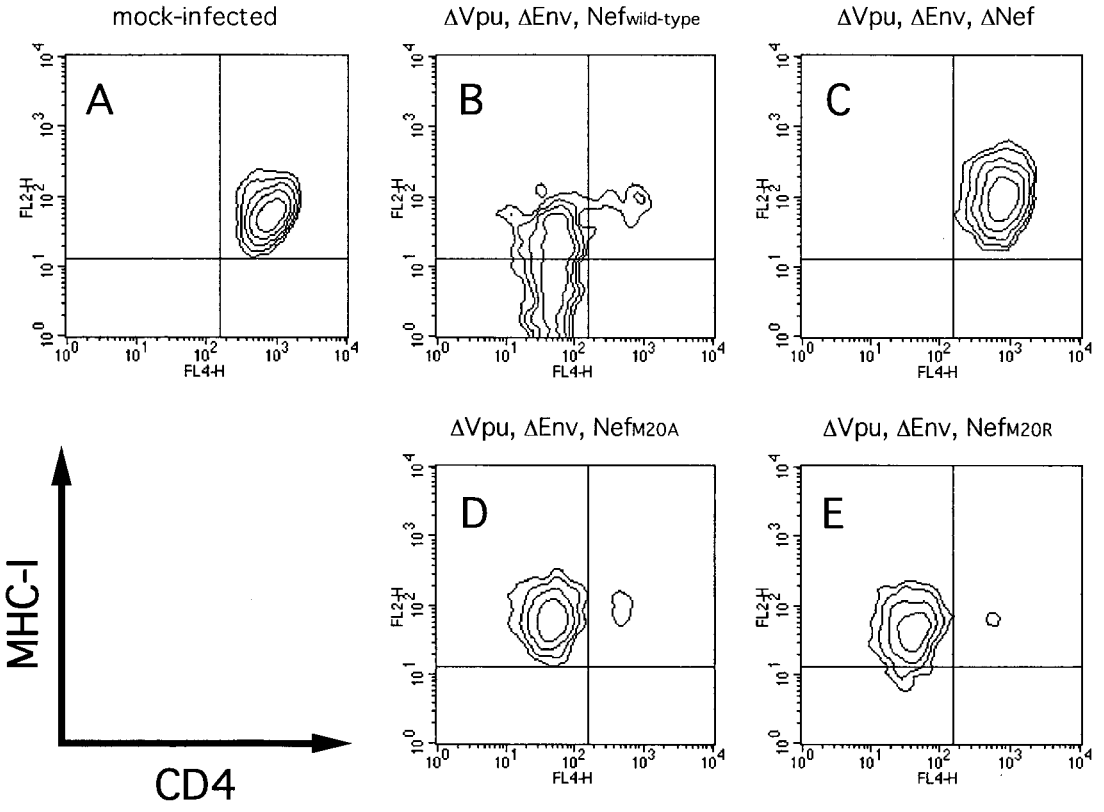

FIG. 5. Effect of mutations in the Nef protein on CD4 down-regulation on the surfaces of cells infected with VSV-G–HIV-1 pseudotyped virus. The pseudotyped viruses were prepared by cotransfection of pCMV-G and pNL43-Ude1.K1 (B), pNL43-Ude1.K1.nM1T (C), pNL43-Ude1.K1.nM20A (D), or pNL43-Ude1.K1.nM20R (E) into HeLa cells. CEM-GFP cells (10^5) were infected with 2.5×10^7 cpm of RT from each pseudotyped virus, and 2 days later, cells were treated at 4°C for 1 h with RPE-labeled anti-MHC-I antibody and allophycocyanin-labeled anti-CD4 antibody. The cells were then washed, fixed with 1% formaldehyde, and analyzed for fluorescence intensity for CD4 and MHC-I in the cell population expressing GFP by flow cytometry.

typed viruses were then prepared by cotransfection of VSV-G expression vector pCMV-G (33) and the *nef* mutants into HeLa cells as previously described (4). CEM-GFP cells (10^5) were infected with 2.5×10^7 cpm of RT from various pseudotyped viruses, and after 2 days, the cells were stained with RPE-conjugated W6/32 and allophycocyanin-conjugated anti-CD4 (Leu-3A; Becton Dickinson) monoclonal antibodies. The fluorescence intensity for CD4 and MHC-I on the cells expressing GFP was then determined as described above. As can be clearly seen in Fig. 5, the pNL-M20A and pNL-M20R mutants retained the ability to down-regulate CD4 expression while failing to down-modulate the MHC-I level.

The present findings demonstrate that the down-regulation of MHC-I by Nef is a property genetically and functionally separate from virion incorporation of Nef and enhancement of viral infectivity by Nef. It has been shown that the N-terminal alpha-helix region is the determinant for these three effects (6, 20, 29). However, we showed that mutations in Met 20 cause the loss of the ability of Nef to modulate MHC-I expression but not the other effects (Fig. 2 to 4). We also showed that the mutations in Met 20 did not affect the ability of Nef to down-regulate the CD4 level (Fig. 5). Our results support and extend the report of Le Gall et al. on Nef (19) that shows the functional dissociation of MHC-I down-regulation from enhancement of viral infectivity. It is surprising that the region governing MHC-I down-regulation is proximate to but dissociated functionally from that determining virion incorporation of Nef and enhancement of viral infectivity by Nef. Baur et al. have shown that the N-terminal alpha-helix domain of Nef interacts with Lck and a serine kinase (6). Based on this finding, it is postulated that one of the two kinases is required for association of Nef with MHC-I and the other plays a critical role in virion incorporation of Nef and enhancement of viral infectivity by Nef. If so, it is possible that Lck phosphorylates a tyrosine-based motif of the MHC-I cytoplasmic domain to facilitate association between Nef and MHC-I. Alternatively, it can also be speculated that these kinases do not take part in the modulation of MHC-I expression. Mangasarian et al. have shown that treatment of HIV-1-infected cells with herbimycin A, an inhibitor of Src family protein kinases, does not block MHC-I down-regulation (20), which argues against the former hypothesis. In this case, it is probable that the IRERMRR motif including Met 20 associates with MHC-I and that the far N-terminal region in the alpha-helix domain, at residues 6 to 22, interacts with the kinases. Baur et al. have also shown that deletion of residues 16 to 22 reduces but does not completely abolish Lck binding (6), implying that the deletion maintains the Lck-binding domain but deteriorates affinity between Nef and Lck. This result supports the latter hypothesis. Further study on the functional roles of the kinases associated with the N terminus of Nef is required to prove these hypotheses.

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